IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.	:	10/559,097
Applicants	:	Frankard, Valerie et al.
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Title	:	Transgenic Monocotyledonous Plants Overexpressing a NHX Protein and Having Improved Growth Characteristics and Methods for Making the Same
Group Art Unit	:	1638
Examiner	:	Kumar, Vinod
Customer No.	:	76809

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DECLARATION OF VALERIE FRANKARD

I, DR. VALERIE FRANKARD, declare as follows.

1. My credentials as a Ph.D. Biotechnology expert are illustrated in part by my current appointments. I currently serve as Technology Management Coordinator and Rice Yield Research Manager of CropDesign N.V., a BASF Plant Science Company, and I am one of the co-inventors of the patent application referenced above. Most recently, I served as the Chairman of Plant and Environmental Biotechnology Stream at the 14th European Congress on Biotechnology held in Barcelona, Spain September 13-16, 2009. I am also the author and inventor of numerous papers, patents and patent applications in the plant biotechnology area.

2. As a co-inventor of the above-identified patent application, I have read and I understand not only the specification and claims as amended herewith but the content of each of the prior art references Fukuda et al. (EP 1143002), Wu et al. (Plant Cell Physiol. 39, 885-889, 1998) and Chan et al. (Plant Molecular Biology 22, 491-506). The data submitted below illustrate that the claimed invention according to claims submitted simultaneously to this Declaration and set forth in pertinent part in Paragraph 7,

below, gives new and unexpected results over any prior art of which the Applicant is aware, including Fukuda et al., Wu et al. or Chan et al., none of which discloses or teaches the present invention and none of which foreshadows the new and unexpected results attainable with the claimed invention.

3. The use of a seed-specific promoter in the context of the claimed invention gives new and unexpected results from the use of other promoters. The above-identified application itself provides data for the PRO0090::CDS1608 construct, which is SEQ ID NO: 2 operably linked to a seed-specific promoter. This data appears in the specification Example 3 on pages 29-30. Attached hereto is Annex 1, which provides additional data for SEQ ID NO: 2 operably linked to PRO0151, another seed-specific promoter, which construct also resulted in increased yield when expressed in rice; and for SEQ ID NO: 2 operably linked to PRO0110 (root specific promoter), by contrast a negative effect for yield was observed. In addition, no significant effect on yield, either positive or negative, was observed when SEQ ID NO: 2 was operably linked to PRO0061_2 (promoter active in young expanding green tissues), further exemplifying the importance of the seed-specific promoter in the context of the present invention. For three different seed-specific promoters used in the practice of the claimed invention, therefore, new and unexpected results of increasing yield as measured for example by Harvest Index and Thousand Kernel Weight were achieved whereas shoot-specific and root-specific promoters did not –or even caused negative results. These comparative test results and conclusions are set forth in greater detail in the remainder of this Declaration.

4. Salt toxicity is easy to recognize and involves leaf tips' turning white and wilting, although salt toxicity may be confirmed by other methods such as leaf analysis or EC measurement in the soil to rule out other toxicities. The International Rice Research Institute (IRRI) has published guidance for diagnosing growth problems in rice and other cereals and has published a particular sheet on salinity issues, attached hereto, based on Dobermann, A., et al., Rice, Nutrient disorders and nutrient management, Handbook Series, Potash & Phosphate Institute (PPI), Potash & Phosphate Institute of Canada (PPIC) and International Rice Research Institute." The attached sheet which begins "Salinity" is part of the "RiceDoctor" publication at http://www.knowledgebank.irri.org/RiceDoctor/default.htm as of August 18, 2009. The RiceDoctor gives some practical ranges for electrical conductivity (EC), which are valid for "average" rice varieties, that is, rice varieties that are neither salt tolerant nor salt sensitive: EC <2 dS m-1 optimum, no yield reduction; EC>4 dS m-1 slight yield reduction (10-15%);

EC>6 dS m-1 moderate reduction in growth and yield (20-50%); and EC> 10 dS m-1 >50% yield reduction in susceptible cultivars. The 15mM solution mentioned in Paragraph 6 has an EC of about 3.5 dS m-1, which normally should not give a yield reduction. In comparison, the standard (non-salt) nutrient solution we use has an EC of about 1-1.2 dS m-1.

5. As summarized in the Annex 1 hereto, we repeated Examples 1 and 2 of the above-identified specification with SEQ ID NO 2 operably linked to PRO0151 (seed-specific promoter), with the exception that no second confirmation experiment was carried out. The results of the experiments showed that out of 6 tested lines, 2 had increased Harvest Index (overall increase of 35%, p-value 0.343) and one line had increased Thousand Kernel Weight (TKW) (6.4% increase, p-value 0.117), all compared to nullizigous plants. The harvest index is hereby defined as the ratio between total seed yield and the above ground area (mm^2), multiplied by a factor 10^6. The TKW is the weight of thousand seeds.

6. Also as summarized in the Annex 1 hereto, we repeated Examples 1 and 2 of the above-identified specification with SEQ ID NO: 2 operably linked to PRO0110 (root-specific promoter), although the second confirmation experiment referred to in the specification was not carried out. In particular, two experiments were carried out, one under mild salt stress (15 mM NaCl) and the other under drought stress. Because the salt stress was mild, similar results for yield would have been expected under non-salt stress conditions. Under mild salt stress, as shown on Annex 1, one line had a 34.3% reduction in total number of seeds, two lines had a reduction of 4.1% in TKW, and two lines had a 32.75% reduction in the number of first panicles.

7. In view of the results reported above and on the attached, it is my opinion that the use of the seed specific promoter according to the following claim: *A method for improving plant growth characteristics, comprising increasing, in a monocotyledonous plant, expression of an isolated nucleic acid encoding an Na+H+/ exchanger (NHX) protein according to SEQ ID NO. 2, wherein said plant is grown under non-salt stress conditions, wherein the increasing expression is effected by introducing and expressing in the plant said nucleic acid having the sequence according to SEQ ID NO. 1 in the sense orientation under the control of a seed-specific promoter, and wherein said growth characteristic is increased yield/biomass and/or modified plant architecture* gives new and unexpected results over

the same method practiced with a root- or shoot-specific promoter. For this reason, and because none of Fukuda et al, Wu et al., or Chan et al. teach or suggest the possibility of new and unexpected results with a seed-specific promoter in particular, the claimed invention is new, useful and nonobvious over the prior art of record (and any other prior art of which I am aware including WO 99/47679, which does not teach the use of a seed-specific promoter to achieve increased yield/biomass or improved plant architecture).

8. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By________________________ date 8 Feb 2010

Dr. Valerie Frankard

Annex 1

Extra data for SEQ ID NO: 2 operably linked to PRO0151 (seed-specific promoter):

Preparation of the construct, generation of transgenic rice plants and evaluation of the plants were as described in Examples 1 and 2 of the specification, with the exception that no second confirmation experiment was carried out.

It was shown that out of 6 tested lines, 2 lines had increased Harvest Index (respectively 34.2% and 36.5%, each with a p-value <0.1) and one line had increased TKW--"the weight of thousand seeds"--(6.4% increase, p-value 0.0002), all compared to nullizigous plants. The harvest index is herein defined as the ratio between the total seed yield and the above ground area (mm^2), multiplied by a factor 10^6. The TKW of plants is derived from the number of filled seeds counted and their total weight.

Extra data for SEQ ID NO: 2 operably linked to PRO0110 (root-specific promoter):

Preparation of the construct, generation of transgenic rice plants and evaluation of the plants were as described in Examples 1 and 2 of the specification, with the exception that no second confirmation experiment was carried out.

Two experiments have been carried out, one under mild salt stress (15 mM NaCl), the other under drought stress. Because the salt stress was mild, similar results for yield would have been expected under non-salt stress conditions.

Mild salt stress:

It was shown that out of 5 tested lines, one line had a reduction in total number of seeds of 34 % (p-value of 0.086), two lines had a reduction in Thousand Kernel Weight (overall decrease of -4%, p-value 0.022) and two lines had a reduction in the number of first panicles (overall decrease -22%, p-value 0.08).

Drought stress:

Out of 4 tested lines, three lines had a reduction in the total number of seeds (overall reduction -9%, p-value 0.061) and two out of four lines showed a decrease in the number of filled seeds (-14.6% with a p-value of 0.186 respectively -21.3% with a p-value of 0.062). The overall decrease was -11.2% (p-value of 0.151).

Salinity

Symptoms | Confirmation | Problems with similar symptoms | Why and where it occurs | Mechanism of damage | When damage is important | Economic importance | Management principles | Source

Whitening and browning of leaves (IRRI)

Diagnostic summary

Effect on plants

- affects respiration and photosynthesis processes
- decreased biological N_2 fixation and soil N mineralization

Signs

- affected leaves with white tips
- some leaves with chlorotic patches
- stunting
- reduced tillering
- patchy field growth

Importance/Occurrence

- important throughout the growth cycle of the rice plant
- associated with poor irrigation practice or insufficient irrigation water, alkaline soils in inland areas, increase in the level of saline groundwater, and intrusion of saline seawater in coastal areas
- may be accompanied by P deficiency, Zn deficiency, Fe deficiency, or B toxicity

Full fact sheet

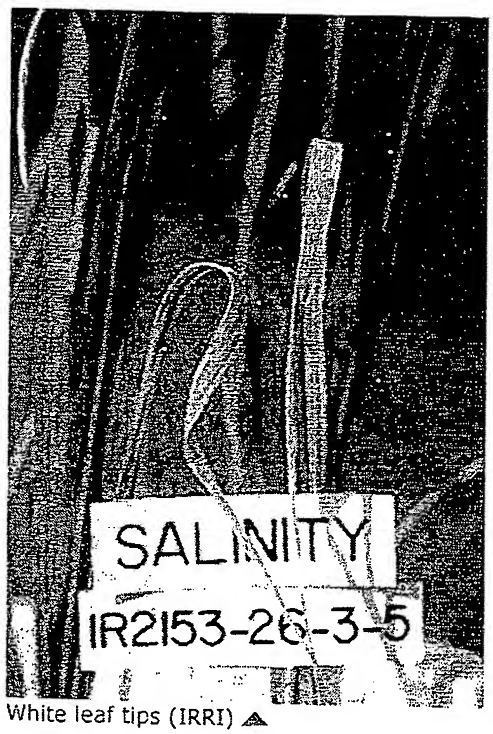
Symptoms

- Tips of affected leaves turn white
- Chlorotic patches appear on some leaves
- Plant stunting and reduced tillering
- Patchy field growth
- Symptoms first manifest themselves in the first leaf, followed by the second, and then in the growing leaf
- Salinity or sodicity may be accompanied by P deficiency, Zn deficiency, Fe deficiency, or B toxicity

Further effects on rice growth:

- Reduced germination rate
- Reduced plant height and tillering
- Poor root growth
- Increased spikelet sterility
- Excess Na uptake decreases 1,000-grain weight and total protein content in grain, but does not alter major cooking qualities of rice
- Decreased biological N_2 fixation and soil N mineralization

Patchy field (IRRI)



White leaf tips (IRRI) ▲

Confirmation

Plant and soil can be tested to confirm salinity.

Increased Na content in rice plants may indicate salinity injury, which may lead to yield loss. The critical concentration of salt (NaCl) in leaf tissue at which toxicity symptoms appear, however, differs widely between varieties. Varieties showing the greatest tolerance for salt within plant tissues are not necessarily those showing the greatest overall phenotypic resistance to salinity.

The correlation between Na:K ratio and salinity tolerance has been established; however, no absolute critical levels in plant tissue are known. A Na:K ratio of <2:1 in the grain may indicate salt-tolerant rice varieties.

The Na:Ca ratio in plant tissue does not seem to be a good indicator of salinity. No effects on growth or NaCl concentration in the shoot were found over the range of Na:Ca ratios (5-25:1) commonly found in the field.

On soil, EC in saturation extract or soil solution: For rice growing in flooded soil, EC is measured in the soil solution or in a saturation extract (EC_e). For upland rice grown at field capacity or below, EC in soil solution is about twice as great as that of the saturation extract. A rough approximation of the yield decrease caused by salinity is:

Relative yield(%) = 100 - [12(EC_e - 3)]

- EC_e <2 dS m-1 optimum, no yield reduction
- EC_e >4 dS m-1 slight yield reduction (10-15%)
- EC_e >6 dS m-1 moderate reduction in growth and yield (20-50%)
- EC_e >10 dS m-1 >50% yield reduction in susceptible cultivars

Exchangeable Na percentage (ESP):

- ESP <20% no significant yield reduction
- ESP >20-40% slight yield reduction (10%)
- ESP >80% 50% yield reduction

Sodium adsorption ratio (SAR):

- SAR >15 sodic soil (measured as cations in saturation extract)

Irrigation water has:

- pH 6.5-8, EC <0.5 dS m^{-1} high-quality irrigation water
- pH 8-8.4, EC 0.5-2 dS m^{-1} medium- to bad-quality irrigation water
- pH >8.4, EC >2 dS m^{-1} unsuitable for irrigation
- SAR <15 high-quality irrigation water, low Na
- SAR 15-25 medium- to bad-quality irrigation water, high Na
- SAR >25 unsuitable for irrigation, very high Na

Notes:

- Measurement of EC as an indicator of salinity is rapid and simple. EC alone, however, is insufficient to assess the effects of salinity on plant growth because salt concentrations at the root surface can be much greater than in the bulk soil. In addition, EC only measures the total salt content, not its composition. Na and B must be considered as well. Salinity is highly variable in the field,

both between seasons and within individual fields. Individual EC values must be treated with caution unless they are based on representative soil samples.

- From EC, the osmotic potential of the saturation extract can be estimated as:
  - Osmotic potential (MP_a) = EC × 0.036
- If the samples do not contain much gypsum, EC measurements can be converted as follows:
  - EC_e = 2.2 × EC1:1 EC1:1 measured in 1:1 soil:water suspension
  - EC_e = 6.4 × EC1:5 EC1:5 measured in 1:5 soil:water suspension

Problems with similar symptoms

No other deficiency exhibits these symptoms but salinity.

Why and where it occurs

Plant growth on saline soils is mainly affected by high levels of soluble salts (NaCl) causing ion toxicity, ionic imbalance, and impaired water balance. On sodic soils, plant growth is mainly affected by high pH and high HCO_3- concentration. The major causes of salinity or sodicity are as follows:

- Poor irrigation practice or insufficient irrigation water in seasons/years with low rainfall.
- High evaporation. Salinity is often associated with alkaline soils in inland areas where evaporation is greater than precipitation.
- An increase in the level of saline groundwater.
- Intrusion of saline seawater in coastal areas (e.g., Mekong Delta, coastal India)

Salt-affected soils (~11 million ha in South and Southeast Asia) are found along coastlines or in inland areas where evaporation is greater than precipitation. Salt-affected soils vary in their chemical and physical properties, but salinity is often accompanied by P and Zn deficiency, whereas Fe toxicity is common in acid sulfate saline soils.

Salt-affected soils can be grouped into:

- saline soils (EC >4 dS m^{-1}, ESP <15%, pH <8.5)
- saline-sodic soils (EC 4 dS m^{-1}, ESP >15%, pH ~8.5)
- sodic soils (EC <4 dS m^{-1}, ESP >15%, pH >8.5, SAR >15)

Examples of salt-affected soils include:

- saline coastal soils (widespread along coasts in many countries)
- saline acid sulfate soils (e.g., Mekong Delta, Vietnam)

- neutral to alkaline saline, saline-sodic, and sodic inland soils (e.g., India, Pakistan, Bangladesh)
- acid sandy saline soils (Korat region of northeast Thailand)

Mechanism of damage

Salinity is defined as the presence of excessive amounts of soluble salts in the soil (usually measured as electrical conductivity, EC). Na, Ca, Mg, Cl, and SO_4 are the major ions involved. Effects of salinity on rice growth are as follows:

- Osmotic effects (water stress)
- Toxic ionic effects of excess Na and Cl uptake
- Reduction in nutrient uptake (K, Ca) because of antagonistic effects

The primary cause of salt injury in rice is excessive Na uptake (toxicity) rather than water stress, but water uptake (transpiration) is reduced under high salinity. Plants adapt to saline conditions and avoid dehydration by reducing the osmotic potential of plant cells. Growth rate, however, is reduced. Antagonistic effects on nutrient uptake may occur, causing deficiencies, particularly of K and Ca under conditions of excessive Na content. For example, Na is antagonistic to K uptake in sodic soils with moderate to high available K, resulting in high Na:K ratios in the rice plant and reduced K transport rates.

Sodium-induced inhibition of Ca uptake and transport limits shoot growth. Increasing salinity inhibits nitrate reductase activity, decreases chlorophyll content and photosynthetic rate, and increases the respiration rate and N content in the plant. Plant K and Ca contents decrease but the concentrations of NO^{3-}-N, Na, S, and Cl in shoot tissue increase. Rice tolerates salinity during germination, is very sensitive during early growth (1-2-leaf stage), regains tolerance during tillering and elongation, but becomes sensitive again at flowering.

Several factors affect the tolerance of different rice varieties to salinity:

- Transpiration rate and potential for osmotic adjustment.
- Differences in nutrient uptake under Na stress. Tolerant cultivars have a narrower Na:K ratio (higher K uptake) and greater leaf Ca^{2+} content than susceptible cultivars.
- Efficient exclusion of Na^+ and Cl^-. Salt-tolerant rice varieties have a reduced Na^+ and Cl- uptake compared with less tolerant cultivars.
- Rapid vegetative growth results in salt dilution in plant tissue.

When damage is important

Rice is more tolerant of salinity at germination, but plants may become affected at transplanting, young seedling, and flowering stages. Thus, this problem occurs throughout the growth cycle of the rice crop.

Economic importance

Salinity can be a major problem in localized areas - tending to occur in low coastal regions and semi-arid inland saline areas.

Management principles

Varieties that tolerate salinity are available, but their use does not substitute for proper water and irrigation management. Breeders will unlikely be able to produce varieties with ever-increasing tolerance of salinity. A variety adapted to present levels of salinity may not survive if salinity increases because water management practices have not been corrected. Rice is a suitable crop for the reclamation of both sodic and saline soils. On sodic soils, rice cultivation results in a large cumulative removal of Na caused by mobilization of insoluble $CaCO_3$. On saline soils, cultivation practices lead to the loss of salts by leaching. Management of salinity or sodicity must include a combination of measures. Major choices include the following:

- **Cropping system:** In rice-upland crop systems, change to double-rice cropping if sufficient water is available and climate allows. After a saline soil is leached, a cropping pattern that includes rice and other salt-tolerant crops (e.g., legumes such as clover or *Sesbania*) must be followed for several years.
- **Varieties:** Grow salt-tolerant varieties (e.g., Pobbeli, Indonesia; IR2151, Vietnam; AC69-1, Sri Lanka; IR6, Pakistan; CSR10, India; Bicol, Philippines). This is a short-term solution that may result in increased salinity over the longer term if other amelioration measures are not implemented.
- **Seed treatment**: In temperate climates where rice is direct seeded, coat seed with oxidants (e.g., Ca peroxide at 100% of seed weight) to improve germination and seedling emergence by increased Ca and O2 supply. Alternatively, treat rice seeds with CaCl2 to increase seed Ca2+ concentration.
- **Water management:** Submerge the field for two to four weeks before planting rice. Do not use sodic irrigation water or alternate between sodic and nonsodic irrigation water sources. Leach the soil after planting under intermittent submergence to remove excess salts. Collect and store low saline rainwater for irrigation of dry-season crops (e.g., by establishing reservoirs). In coastal areas, prevent intrusion of salt water.
- **Fertilizer management:** Apply Zn (5-10 kg Zn ha^{-1}) to alleviate Zn deficiency. Apply sufficient N, P, and K. The application of K is critical because it improves the K:Na, K:Mg, and K:Ca ratios in the plant. Use ammonium sulfate as N source and apply N as topdressing at critical growth stages (basal N is used less efficiently on saline and sodic soils). In sodic soils, the replacement of Na by Ca (through the application of gypsum) may reduce P availability and result in an increased requirement for P fertilizer.
- **Organic matter management:** Organic amendments facilitate the reclamation of sodic soils by increasing the partial CO_2 pressure and decreasing pH. Apply rice straw to recycle K. Apply farmyard manure.

The following are options for treatment of salinity:

- **Saline soils:** Salinity can only be reduced by leaching with salt-

free irrigation water. Because rice has a shallow root system, only the topsoil (0-20 cm) requires leaching. Cost, availability of suitable water, and soil physical and hydraulic characteristics determine the feasibility of leaching. To reduce the level of salinity in affected soils, electrical conductivity in the irrigation water should be <0.5 dS m^{-1}). Where high-quality surface water is used (EC ~0), the amount of water required to reduce a given EC_e to a critical-level EC_c can be calculated as follows:

- $A_{iw}=A_{sat[}(EC_e\ /ECc)+1]$
- where A_{iw} represents the amount of irrigation water (in cm) added during irrigation and A_{sat} is the amount of water (cm) in the soil under saturated conditions. For example, to lower an initial EC_e of 16 dS m^{-1} to 4 dS m^{-1} in the top 20 cm of a clay loam soil (A_{sat} = 8-9 cm), about 40 cm of fresh water is required. Subsurface drains are required for leaching salts from clay-textured soils.

- **Sodic soils:** Apply gypsum (CaSO4) to reduce Na saturation of the soil (ESP, Na:K ratio). Because of complex chemical and physical interactions, it is difficult to calculate the exact amount of gypsum required. The amount of Ca^{2+} contained in gypsum required to reduce the ESP to a target level can be estimated as follows:
  - Ca (kg ha-1) = $(ESP_0 - ESP_d) \times CEC \times B \times D \times 20.04$
  - where ESP_0 is the original and ESP_d is the target ESP value (% of CEC), CEC is in cmolc kg^{-1}, B is the bulk density (g cm^{-3}), and D is the soil depth (m) to be reclaimed.
- Foliar application of K, particularly if a low-tolerance variety is grown on saline soil. Spray at the late tillering and panicle initiation stages.

Source

Dobermann A, Fairhurst T. 2000. Rice. Nutrient disorders & nutrient management. Handbook series. Potash & Phosphate Institute (PPI), Potash & Phosphate Institute of Canada (PPIC) and International Rice Research Institute. 191 p.

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